United States Department of Agriculture
Center for Veterinary Biologics
Testing Protocol

SAM 110

Supplemental Assay Method for Titration of Eastern, Western, and Venezuelan Equine Encephalomyelitis Virus Neutralizing Antibodies

Date:	December 10, 2004
Supersedes:	MVSAM0110.01, Dated February 17, 1998
Number:	VIRSAM0110.02
Standard Requirement:	9 CFR 113.207
Contact:	Kenneth A. Eernisse, (515) 663-7857 Peg A. Patterson, (515) 663-7334

Approvals:

/s/Donna M. Gatewood_____ Date: 12/16/04
Donna M. Gatewood, Section Leader
Virology Section

/s/Richard E. Hill, Jr._____ Date: 16-Dec-04
Richard E. Hill, Jr., Director
Policy, Evaluation, and Licensing
Center for Veterinary Biologics

/s/Rebecca L.W. Hyde_____ Date: 17Dec04
Rebecca L.W. Hyde, Section Leader
Quality Assurance
Center for Veterinary Biologics

United States Department of Agriculture
Animal and Plant Health Inspection Service
P. O. Box 844
Ames, IA 50010

Supplemental Assay Method for Titration of Eastern, Western, and Venezuelan Equine Encephalomyelitis Virus Neutralizing Antibodies

Table of Contents

Supplemental Assay Method for Titration of Eastern, Western, and Venezuelan Equine Encephalomyelitis Virus Neutralizing Antibodies

1. Introduction

This Supplemental Assay Method (SAM) describes an ***in vitro*** assay method, employing a cell culture system, for determining the neutralizing antibody titers of sera from guinea pigs vaccinated against Eastern (EEE), Western (WEE), and/or Venezuelan (VEE) Equine Encephalomyelitis viruses.

2. Materials

2.1 Equipment/instrumentation

2.1.1 Pipetters:[1] 20-µl and 200-µl

2.1.2 Blender[2]

2.1.3 1000-ml borosilicate glass media bottle with screw-top lid[3]

2.1.4 36°± 2°C, 5% ± 1% CO_2, 70-80% humidity incubator[4]

2.1.5 Water bath[5]

2.1.6 Vortex mixer[6]

2.2 Reagents/supplies

2.2.1 Vero 76 cell culture free of extraneous agents as tested by the Code of Federal Regulations, Title 9 (9CFR)

2.2.2 Growth Medium

2.2.2.1 1000 ml MEM[7]

2.2.2.2 Sterilize through 0.22-µm filter[8]

1 Pipetman, Rainin Instrument Co., Mack Rd., Box 4026, Woburn, MA 01888 or equivalent
2 Waring blender, Cat. no. 14-509-35, Fisher Scientific, Inc., or equivalent
3 Media bottle, Cat. no. 219760, Wheaton Scientific, 1000 N. 10th St., Millville, NJ 08332 or equivalent
4 Incubator, Model 3158, Forma Scientific, Inc., Box 649, Marietta, OH 45750-0649 or equivalent
5 Water bath, Cat. no. 15-461-10, Fisher Scientific, Inc., or equivalent
6 Vortex-2 Genie, Model G-560, Scientific Industries, Inc., 700 Orville Dr., Bohemia, NY 11716 or equivalent
7 MEM with Earle=s salts without sodium bicarbonate, Cat. no. 410-1500EF, Life Technologies, Inc., 8400 Helgerman Ct., Gaithersburg, MD 20884 or equivalent
8 Disposable filter, Cat. no. 12122, Gelman Sciences, 600 S. Wagner Rd., Ann Arbor, MI 48106 or equivalent

2.2.2.3 Aseptically add:

1. 10 ml L-glutamine[9]

2. 5 ml lactalbumin hydrolysate or edamine[10]

3. 100 units/ml penicillin[11]

4. 50 µg/ml gentamicin sulfate[12]

5. 100 µg/ml streptomycin[13]

6. 2.5 µg/ml amphotericin B[14]

7. 100 ml gamma-irradiated fetal bovine serum (FBS)

2.2.2.4 Store at 2°- 7°C

2.2.3 Diluent Medium

2.2.3.1 1000 ml MEM

2.2.3.2 2.2 g sodium bicarbonate[15]

2.2.3.3 Sterilize through 0.22-µm filter

2.2.3.4 Aseptically add:

1. 10 ml L-glutamine

2. 5 ml lactalbumin hydrolysate or edamine

3. 100 µg/ml streptomycin

4. 100 units/ml penicillin

9 L-glutamine-200mM (100X), liquid, Cat. no. 320-503PE, Life Technologies, Inc., or equivalent
10 Edamine S, Cat. no. 59102, Sheffield Products, P.O. Box 630, Norwick, NY 13815 or equivalent
11 Penicillin solution, Schering Laboratories, 2000-T Galloping Hill Rd., Kenilworth, NJ 07033 or equivalent
12 Gentocin solution, Schering Laboratories or equivalent
13 Streptomycin solution, Schering Laboratories or equivalent
14 Fungizone, E.R. Squibb & Sons, Inc., 1 Squibb Dr., Cranberry, NJ, 08512 or equivalent
15 Cat. no. S 5761, Sigma Chemical Co., P.O. Box 14508, St. Louis, MO 63178 or equivalent

5. 50 µg/ml gentamicin sulfate

6. 2.5 µg/ml amphotericin B

2.2.3.5 Store at 2°- 7°C

2.2.4 2% Diluent Medium

2.2.4.1 100 ml Diluent Medium

2.2.4.2 2 ml FBS

2.2.4.3 Store at 2°- 7°C

2.2.5 2X Medium

2.2.5.1 100 ml 10X MEM

2.2.5.2 2.2 g sodium bicarbonate

2.2.5.3 340 ml deionized water (DI)

2.2.5.4 Sterilize through 0.22-µm filter

2.2.5.5 Aseptically add:

1. 2% of 7.5% sodium bicarbonate

2. 5 ml lactalbumin hydrolysate or edamine

3. 100 units/ml penicillin

4. 50 µg/ml gentamicin sulfate

5. 100 µg/ml streptomycin

6. 2.5 µg/ml amphotericin B

7. 50 ml gamma-irradiated FBS

2.2.5.6 Store at 2°- 7°C

2.2.6 2% Tragacanth Gum (Trag)

2.2.6.1 20 g Trag[16]

2.2.6.2 1000 ml DI

2.2.6.3 Mix small amounts at a time vigorously with a blender set on high

2.2.6.4 Pour 500 ml each into 1000-ml media bottles

2.2.6.5 Sterilize by autoclaving for 30 minutes

2.2.6.6 Store at 2°- 7°C

2.2.7 7.5% Sodium Bicarbonate

2.2.7.1 7.5 g $NaHCO_3$

2.2.7.2 Q.S. to 100 ml with DI.

2.2.7.3 Sterilize through a 0.22-µm filter.

2.2.7.4 Store at room temperature.

2.2.8 Overlay Medium

2.2.8.1 Mix equal volumes of 2X Medium and 2% Trag

2.2.8.2 Store at 2°- 7°C

2.2.9 70% Ethyl Alcohol

2.2.9.1 74 ml ethyl alcohol[17]

2.2.9.2 26 ml DI

2.2.9.3 Store at room temperature

2.2.10 Crystal Violet Stain

2.2.10.1 7.5 g crystal violet[18]

16 Acros AC42138-5000, Fisher Scientific, Inc., or equivalent
17 Ethyl alcohol denatured 190 proof, Cat. no. 7018, Mallinckrodt Inc., 222 Red School Lane, Phillipsburg, NJ 08865 or equivalent
18 Cat. no. C 0775, Sigma Chemical Co., or equivalent

2.2.10.2 50 ml 70% ethyl alcohol

2.2.10.3 250 ml formaldehyde[19]

2.2.10.4 Q.S. to 1000 ml with DI

2.2.10.5 Dissolve crystal violet in alcohol, add remaining ingredients, filter through filter paper[20].

2.2.10.6 Store at room temperature

2.2.11 EEE, WEE, and VEE Indicator Viruses[21]

2.2.12 Tissue culture plates, 6-well[22]

2.2.13 12 x 75-mm polystyrene tubes[23]

2.2.14 Pipette[24]

3. Preparation for the test

3.1 Personnel qualifications/training

Personnel shall have received the required number of vaccinations against EEE and WEE and have evidence of serological protection. Personnel shall have sufficient training in standard laboratory procedures.

3.1.1 All work shall be performed in a Biosafety Level 3 room when live indicator virus is present.

3.1.2 Live indicator virus shall be collected in suitable containers for inactivation by autoclaving. Fluids from plate cultures are treated with crystal violet/formalin stain, and should be disposed of in accordance with Biosafety Level 3 agent disposal procedures.

19 Cat. no. F79, Fisher Scientific, Inc., or equivalent
20 Whatman #1, Cat. no. 1001, Fisher Scientific, Inc., or equivalent
21 Available upon request from the Center for Veterinary Biologics-Policy, Evaluation, and Licensing (CVB-PEL), P.O. Box 844, 1800 Dayton Ave., Ames, IA 50010
22 Falcon 3046, Becton Dickinson Labware, Becton Dickinson & Co., 2 Bridgewater Lane, Lincoln Park, NJ 07035 or equivalent
23 Falcon 2058, Becton Dickinson Labware or equivalent
24 Falcon®, Becton Dickinson Labware or equivalent

3.2 Preparation of equipment/instrumentation

3.2.1 Set the water bath at 56°± 2°C for serum inactivation.

3.2.2 Set the water bath at 36°± 2°C to warm the Overlay Medium.

3.3 Preparation of reagents/control procedures

3.3.1 Preparation of Vero 76 cell cultures

3.3.1.1 Multiple 6-well plates are seeded with Vero 76 cells, in Growth Medium, at a cell count that will produce a monolayer after one day of incubation at 36°± 2°C. (Five plates are needed for each virus, 1 plate for Trag control, and 1 plate for cell control.) Cells older than three days should not be used in the test. Growth Medium is changed if excess acidity of the medium is observed as indicated by a change from red to yellow of Growth Medium or cells are not confluent two days after seeding.

3.3.2 Indicator Virus Dilution. A vial of Indicator Virus is thawed with warm tap water and diluted in 2% Diluent Medium to contain 60-200 plaque forming units (PFU) per 0.1 ml. This is the Working Dilution of the Indicator Virus.

3.3.2.1 An Indicator Virus Control is run at the time of the plaque reduction assay to determine the plaque count.

1. The Working Dilution of each Indicator Virus is mixed with an equal volume of Diluent Medium to represent the same dilution used with the guinea pig/virus mixture.

3.4 Preparation of the sample

3.4.1 Dilution of vaccinated guinea pig sera (VGPS) for EEE and WEE serum neutralization (SN).

3.4.1.1 Heat inactivate VGPS in a 56°± 2°C water bath for 30 minutes ± 5 minutes.

3.4.1.2 Pipette 190 µl of Diluent Medium into ten labeled tubes.

3.4.1.3 Pipette 10 µl of serum from each VGPS into the tubes and vortex. This is a 1:20 dilution of the sera.

3.4.2 Dilution of control guinea pig sera (CGPS) and VGPS for VEE SN.

3.4.2.1 Heat inactivate sera in a 56°± 2°C water bath for 30 ± 5 minutes.

3.4.2.2 Pipette 100 µl of Diluent Medium into two labeled tubes for CGPS dilutions or ten labeled tubes for VGPS dilutions.

3.4.2.3 Pipette 100 µl of serum from each CGPS or VGPS into the tubes and vortex. This is a 1:2 dilution of the sera.

4. Performance of the test

4.1 Add 200 µl of the Working Dilution of an Indicator Virus (**Section 3.3.2**) to each labeled tube of diluted sera and vortex. This increases the serum dilution to 1:40 for the EEE and WEE SN and 1:4 for the VEE SN.

4.2 Incubate at 36°± 2°C for 60 ± 10 minutes.

4.3 Pour off the medium from the plates containing Vero 76 cells.

4.4 Inoculate 2 wells/sample with 100 µl/well of each virus-serum mixture.

4.5 Inoculate 100 µl/well of the Indicator Virus Control mixture (**Section 3.3.2.1**) into each of 6 wells/Indicator Virus.

4.6 Maintain 2 or more wells as uninoculated cell culture controls.

4.7 Incubate inoculated plates at 36°± 2°C for 60 ± 10 minutes to allow for virus adsorption.

4.8 Add 3.0 ml/well of Overlay Medium (**Section 2.2.7**) to the plates. Discard any unused, warmed Overlay

Medium.

4.9 Incubate the WEE plates undisturbed at 36°± 2°C for 2 days. Incubate the EEE and VEE plates undisturbed at 36°± 2°C for 3 days.

4.10 At the end of incubation, without removing Overlay Medium, pipette 3 ml of the Crystal Violet Stain (**Section 2.2.9**) into each well of the plates using the repetitive syringe.

4.11 Allow plates to stand at room temperature overnight.

4.12 Wash the Crystal Violet Stain from the cell monolayers by dipping each plate several times in a container of running cold tap water. Allow to air dry.

4.13 PFU counting

4.13.1 The PFU are visible as clear, circular areas in the cell monolayer where the cells have been destroyed by the virus.

4.13.2 Count the number of PFU for each well.

4.13.2.1 Average the number of PFU between the duplicate wells for each VGPS and CGPS sample on each Indicator Virus.

4.13.2.2 Average the number of PFU between the 6 wells of the Indicator Virus Control wells.

5. Interpretation of the test results

5.1 The average number of PFU from the 6 wells of the Indicator Virus Control is divided by 2 to obtain the 50% plaque reduction count. For example, if the average PFU count for EEE Indicator Virus is 58, the 50% plaque reduction count for EEE would be 29.

5.2 For a valid assay, the Indicator Virus Control must have an average PFU count between 30-100.

5.3 For a valid assay, the CGPS must have a titer <1:4 against each Indicator Virus. CGPS samples with

PFU above the 50% plaque reduction count have a titer <1:4 for that Indicator Virus.

5.4 Compare the average PFU for each VGPS to the 50% plaque reduction count. VGPS samples with PFU below the 50% plaque reduction count have a titer ≥1:40 for EEE or WEE or a titer ≥1:4 for VEE.

6. Report of test results

6.1 Test results are reported as the number of VGPS having a titer ≥1:40 for EEE or WEE or a titer ≥1:4 for VEE (e.g. 9/10 ≥1:40). CGPS are reported as the number having a titer <1:4 (e.g. 2/2 <1:4).

6.2 Record all test results on the test record (worksheet).

7. References

7.1 Code of Federal Regulations, Title 9, Part 113.207.

7.2 Katz JB, SK Hanson. Encephalomyelitis vaccines: a Vero-derived cell culture alternative to primary duck embryonic cell cultures. 1988. Vaccine 6:6.

7.3 Richmond JY, RW McKinney, ed. Biosafety in Microbiological and Biomedical Laboratories. Centers for Disease Control and Prevention. Third edition. 1993. U.S. Government Printing Office.

8. Summary of revisions

This document was revised to meet the current CVB Quality Assurance requirements, to clarify the practices currently in use in the CVB-PEL, and to provide additional detail.

Supplemental Assay Method for Titration of Eastern, Western, and Venezuelan Equine Encephalomyelitis Virus Neutralizing Antibodies

While no significant changes impacting the test were made from the previous protocol, the following changes were made to the document:

- **2.1.1** Deleted self-refilling syringe
- **2.2.2.3/2.2.5.5** Changed heat-inactivated FBS to gamma-irradiated serum
- **3.1** Added WEE and need to have evidence of serological protection.
- **3.1.2** Changed statement for disposal of live virus to conform to Biosafety Level 3 guidelines
- **3.3.1** Added amount of plates needed
- **3.3.2.1** Added to represent the same dilution as the guinea pig/virus mixture
- **4.9** Changed 48 hours to 2 days and 72 hours to 3 days
- **5.1** Expressed that the PFU average comes from the 6 wells of the Indicator virus.
- **6.1** Added examples
- The refrigeration temperatures have been changed from 4°± 2°C to 2°- 7°C. This reflects the parameters established and monitored by the Rees system.